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The effect of Na^+ and K^+ on the thermal denaturation of $Na^+ + K^+$-dependent ATPase

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To increase our understanding of the physical nature of the Na^+ and K^+ forms of the $Na^+ + K^+$-dependent ATPase, thermal-denaturation studies were conducted in different types of ionic media. Thermal-denaturation measurements were performed by measuring the regeneration of ATPase activity after slow pulse exposure to elevated temperatures. Two types of experiments were performed. First, the dependence of the thermal-denaturation rate on Na^+ and K^+ concentrations was examined. It was found that both cations stabilized the pump protein. Also, K^+ was a more effective stabilizer of the native state than was Na^+. Secondly, a set of thermodynamic parameters was obtained by measuring the temperature-dependence of the thermal-denaturation rate under three ionic conditions: 60mM-K^+, 150mM-Na^+ and no Na^+ or K^+. It was found that ion-mediated stabilization of the pump protein was accompanied by substantial increases in activation enthalpy and entropy, the net effect being a less-pronounced increase in activation free energy.

A large body of experimental evidence indicates that the $Na^+ + K^+$-dependent ATPase (EC 3.6.1.3) assumes different conformation states that are modulated by ligand concentrations. Furthermore, it is proposed that transitions occur between the different conformational states in a manner that results in ATP-hydrolysis-coupled ion translocation (Karlish *et al.*, 1978; Jørgensen, 1975). Experiments examining the effect of Na^+ and K^+ on protein fluorescence (Karlish *et al.*, 1978; Karlish & Yates, 1978) and proteolytic-digestion patterns (Jørgensen, 1975) have led to the definition of E_1 and E_2 conformational states. The E_1 and E_2 states can be promoted respectively by the presence of Na^+ or K^+, and are thus frequently referred to as the Na^+ and K^+ forms of the enzyme, or as the $E_1 \cdot Na^+$ and $E_2 \cdot K^+$ states.

In order to increase our understanding of the physical nature of the Na^+ and K^+ forms of the pump protein, thermal-denaturation studies conducted in different types of ionic media are reported in the present communication. Two types of experiments are described. First, the dependence of the thermal-denaturation rate on Na^+ and K^+ concentrations is examined at a fixed temperature. Secondly, a set of thermodynamic parameters is obtained by measuring the temperature-dependence of the thermal-denaturation rate under three ionic conditions: 60mM-K^+, 150mM-Na^+ and no Na^+ or K^+.

Experimental

All chemicals were reagent grade. Heparin was obtained from Upjohn Co. (Kalamazoo, MI, U.S.A.), Trasylol from Mobay Chemical Co. (New York, NY, U.S.A.) and Brij 58 from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Unless otherwise specified, all procedures were performed at 0–4°C. Fresh pig kidneys were obtained from a local slaughterhouse and profused, with a syringe, via the renal artery with 150ml/kidney of an ice-cold profusion medium [containing (final concentrations) 250mM-sucrose, 100mM-NaCl, 20mM-KCl, 2.5mM-$MgCl_2$, 1mM-EGTA, heparin (2 i.u./ml), Trasylol (50 kallikrein-inhibitory units/ml) and 10 mM-imidazole/HCl buffer, pH 7.4] within minutes after animal death. Microsomal fractions were isolated by using a variation of the procedure of Jørgensen (1974). Kidney outer medullas were removed, yielding approx. 4g wet wt. of material per kidney, and homogenized at low speed in 10ml of profusion medium/g of tissue in a Polytron homogenizer. The resulting suspension was centrifuged at 6000*g* for 15min. The supernatant was then centrifuged at 48000*g* for 30min. The

pellets were pooled and resuspended in 5 ml of isolation medium [containing (final concentrations) 250 mM-sucrose, 1 mM-EDTA, 0.5 mM-dithiothreitol, Trasylol (50 kallikrein-inhibitory units/ml) and 30 mM-Tris/HCl buffer, pH 7.4]/g of original tissue and centrifuged at 48000 *g* for 30 min. The last resuspension–centrifugation step involving isolation medium was performed three times. The resulting pellets were stored in isolation medium at 3°C. ATPase activity was stable for at least 2 weeks when the preparation was stored at 3°C in isolation medium.

Protein and phosphate determinations respectively utilized the procedures of Peterson (1977) and Bartlett (1959). Total microsomal protein/phospholipid ratios of 1.09–1.23 mg of protein/mg of phospholipid (assuming an average phospholipid molecular weight of 780) were typically obtained. ATPase activities were measured with a coupled enzyme system (Norby, 1971). The assay medium contained (final concentrations) 130 mM-NaCl, 30 mM-KCl, 2.5 mM-$MgCl_2$, 1 mM-EGTA, 0.1 mM-dithiothreitol, 2.5 mM-Na_2ATP, 40 μM-Brij 58, 4.2 mM-phosphoenolpyruvate, 0.1 mM-NADH, pyruvate kinase [10 units (μmol/min)/ml], lactate dehydrogenase [20 units (μmol/min)/ml], 17.5 mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-ethanesulphonic acid)/NaOH buffer, pH 7.4, with or without 0.1 mM-ouabain, and microsomal fraction (10 μg of protein/ml). The ATPase-containing microsomal fraction was incubated for 15 min at 37°C in assay medium lacking NADH, $MgCl_2$ and ATP to expose the ATP-hydrolysis sites. The assay was then initiated by addition of NADH, ATP and $MgCl_2$. Microsomal fractions isolated and assayed by the methods indicated above typically yielded ATPase activities between 4.0 and 4.5 μmol of ATP hydrolysed/min per mg of total protein at 37°C. Ouabain-insensitive activities were negligible. In each assay 10 μg of total microsomal protein was used. The ATPase assay procedure yielded activity values that typically agreed to within 7.5% when samples were assayed in duplicate.

The endogenous Na^+ and K^+ concentrations of the isolated $Na^+ + K^+$-dependent-ATPase-containing microsomal fractions were not measured. It should be noted that, when assayed in the absence of Na^+ or K^+, ouabain-sensitive ATPase activity was zero.

Thermal-denaturation measurements were performed by monitoring the loss of ATPase activity as a function of incubation time at elevated temperatures. This involved incubating samples in a Haake FL-2 circulating temperature bath for defined periods of time. Samples were then placed in an ice/water slurry to terminate the denaturation reaction. The ATPase activity was subsequently measured as indicated above in this section. The temperature of the water bath was measured with a Fluke 2100-A digital thermocouple, and was found to vary by less than 0.1°C during each series of decay measurements. Sample temperatures were found to be within 0.1°C of bath temperature after 30 s in the temperature bath. The zero-time point was thus defined at 30 s after sample placement in the temperature bath. The placement of samples in the ice/water slurry resulted in an approx. 40°C temperature decrease in 10 s.

Results

An important finding of the present research is that K^+ is more effective than Na^+ at stabilizing the $Na^+ + K^+$-dependent ATPase in a native state. Furthermore, ATPase activity is lost substantially faster in the absence of both Na^+ and K^+ than when one of these cations is included in the medium. These effects are demonstrated in Fig. 1, which depicts thermal-denaturation data for samples containing no Na^+ or K^+, 100 mM-Na^+ and 60 mM-K^+. A more-detailed examination of the effects of Na^+ and K^+ is presented in Fig. 2, in which the dependence of the thermal decay rate on Na^+ and K^+ concentrations is examined. A noteworthy point concerning the data presented in Fig. 2 is that at similar K^+ and Na^+

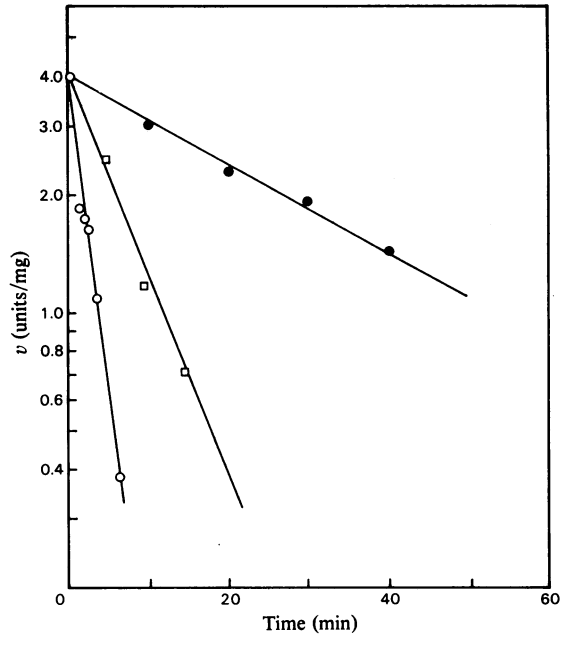

Fig. 1. *Thermal decay of ATPase activity (v) as a function of time for samples with no Na^+ or K^+ (○), 100 mM-Na^+ (□) and 60 mM-K^+ (●), at 59.7°C*

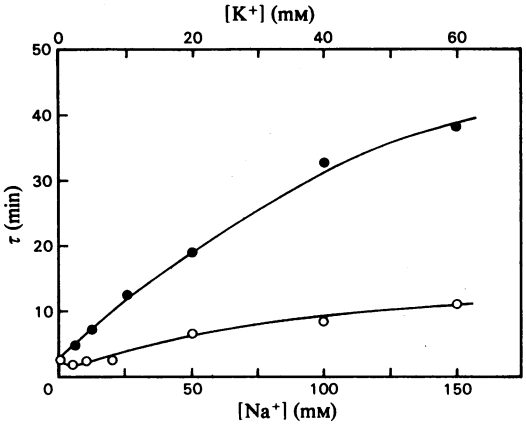

Fig. 2. *Na^+-concentration-dependence* (○, *lower scale*) *and K^+-concentration-dependence* (●, *upper scale*) *of the time constant* (τ) *for the thermal decay of ATPase activity at 59.7°C*

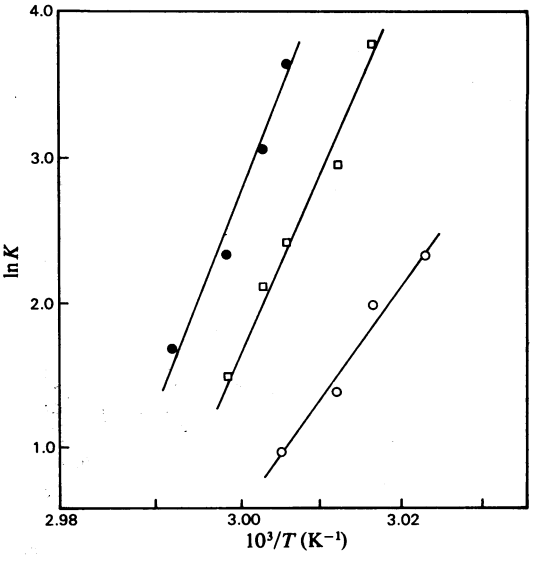

Fig. 3. *Temperature* (T)*-dependence of the thermal decay rate* (K) *of ATPase activity with 150 mM-Na^+* (□), *60 mM-K^+* (●) *and no Na^+ or K^+* (○)

concentrations K^+ is a substantially more effective stabilizer of the native protein state. If the observed stabilization effects were simply due to an increase in ionic strength, equivalent K^+ and Na^+ concentrations would give similar thermal decay rates. This is not the case. In fact, 10 mM-K^+ is more effective at stabilizing the protein than is 150 mM-Na^+.

The results of a second series of experiments, which analyse the effect of temperature change on the thermal decay rate, are presented in Fig. 3. Results obtained from samples that contained no Na^+ or K^+, 150 mM-Na^+ and 60 mM-K^+ are reported. An analysis of the data in Fig. 3 yields the activation entropies ($\Delta S^\ddagger$), enthalpies ($\Delta H^\ddagger$) and free energies ($\Delta G^\ddagger$) contained in Table 1. The calculation of $\Delta G^\ddagger$ and $\Delta S^\ddagger$ utilizes the Eyring approximation (Johnson *et al.*, 1974) that the hypothetical decay rate from the transition state is equal to kT/h, where k is the Boltzmann constant, T is the absolute temperature and h is Planck's constant. The error in $\Delta H^\ddagger$ values reflects the uncertainty in fitting log(rate constant)-versus-$1/T$ data to a straight line. The rate-constant error is the average deviation of the rate constants from the best-fit lines in Fig. 3. The errors in $\Delta G^\ddagger$ and $\Delta S^\ddagger$ values are obtained by propagating the uncertainty in $\Delta H^\ddagger$ and rate-constant values with total differentials. The parameters contained in Table 1 show that ion-mediated stabilization of the protein is accompanied by substantial increases in $\Delta H^\ddagger$ and $\Delta S^\ddagger$, the net effect being a less-pronounced increase in $\Delta G^\ddagger$.

Discussion

A large body of experimental evidence demonstrating the existence of Na^+- and K^+-induced forms of the $Na^+ + K^+$-dependent ATPase is now available. The tryptic-digestion experiments of Jørgensen (1975) show differences in the digestion pattern and the inactivation kinetics when Na^+ or K^+ is included in the digestion medium. Studies utilizing extrinsic and intrinsic fluorescence techniques (Karlish *et al.*, 1978; Karlish & Yates, 1978) demonstrate that Na^+ and K^+ induce different conformational states of the enzyme. Fluorescence measurements also indicate

Table 1. *Thermodynamic parameters for thermal decay of ATPase activity*

Cation concentrations ...	No Na^+ or K^+	150 mM-Na^+	60 mM-K^+
Activation enthalpy (kJ/mol)	670 ± 130	1090 ± 80	1260 ± 80
Activation free energy (kJ/mol)	95.27 ± 0.13	98.62 ± 0.21	101.80 ± 0.25
Activation entropy (kJ/mol)	1670 ± 330	2930 ± 290	3510 ± 290
Absolute temperature (K)	333	333	333
Rate constant (min^{-1})	0.45 ± 0.02	0.134 ± 0.009	0.042 ± 0.004

that, in the absence of ligands, the protein is in the E_1 state (Beaugé & Glynn, 1980). Experiments that investigate the effect of a variety of chemical agents on different properties of the $Na^+ + K^+$-dependent ATPase also support the idea of the existence of $E_1 \cdot Na^+$ and $E_2 \cdot K^+$ conformational states (Hart & Titus, 1973; Skou, 1974; Robinson, 1974). It can thus be assumed with a high degree of certainty that the thermal-denaturation studies reported in the present communication involved enzyme prepared in the $E_1 \cdot Na^+$ and $E_2 \cdot K^+$ states.

The usefulness of thermal-denaturation information as a tool to examine native conformation states is somewhat limited by a lack of understanding of the thermal-denaturation process. Several questions remain unanswered. First, what is the detailed mechanism for the kinetic process leading to a true equilibrium distribution of protein conformation? Secondly, at what stage in the denaturation process is the ability to regenerate activity lost? Finally, what is the nature of the transition state? In particular, is the transition state the same or similar for the denaturation process involving $E_1 \cdot Na^+$ and $E_2 \cdot K^+$ enzyme forms? Similar questions apply to any thermal-denaturation study in which a molecular property is used to monitor the course of denaturation. Despite the preceding unanswered questions, important inferences concerning the thermodynamic state of the $E_1 \cdot Na^+$ and $E_2 \cdot K^+$ conformations can be made if it is assumed that the absolute enthalpies and entropies of the activation complexes are approximately independent of the initial native protein state. When this approximation is made, the conservation laws dictate the following three conclusions. First, the $E_2 \cdot K^+$ state is emphalpically more stable than is the $E_1 \cdot Na^+$ state. Secondly, the $E_1 \cdot Na^+$ state is entropically more stable than is the $E_2 \cdot K^+$ state. This implies that the binding of K^+ restricts the conformational flexibility of the protein to a greater extent than does the binding of Na^+. Thirdly, the net result in terms of free energy is that the $E_2 \cdot K^+$ state is more stable than is the $E_1 \cdot Na^+$ state.

The findings of the present study, that the $Na^+ + K^+$-dependent ATPase is more stable with respect to thermal denaturation in the $E_2 \cdot K^+$ than in the $E_1 \cdot Na^+$ state, and that in absolute terms the $E_2 \cdot K^+$ state might be more stable than the $E_1 \cdot Na^+$ state, is consistent with the results of other studies. Kinetic studies based on the direct monitoring of the E_1–E_2 transition with intrinsic and extrinsic fluorescence (Karlish *et al.*, 1978; Karlish & Yates. 1978) show that the transition from the K^+ to the Na^+ enzyme form is much slower than the conversion from the Na^+ into the K^+ state. Also, the dephosphorylation studies by Mardh (1975) indicate that the E_2–E_1 transition might be slow in the presence of K^+.

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